COMPOSITION AND METHOD FOR DETECTING AND EARLY AND DIFFERENTIATED COUNTING OF GRAM-NEGATIVE MICROORGANISMS.

Technical sector

The present invention is related with the Microbiology field and particularly with a composition and a method for early detection, identification, differentiation and count of microscopic organisms, concretely Gram-negative microorganisms.

Prior Art

The recuperation, identification and count of Gram-negative microorganisms, such as *Salmonella*, *E. coli* and coliforms group, are of a great interest in the clinical diagnosis and in the sanitary quality control of waters, foods and environmental samples.

For the identification and count of the microscopic Gram-negative organisms, exists a range of culture media with formulations that could be considered "traditional", many of them developed from the past century. Within these media could be marked S.S. Agar, S.S. Agar (modified), XLD Medium, Hektoen Enteric Agar, Kristensen Agar and Brilliant Green Agar, used for the identification of *Salmonella* (Soria Melquizo, F. Difco Handbook. Tenth edition. 1984; MERCK Handbook of Culture Media. 1990; OXOID Handbook of Culture Media. 1995)

In general, these media have an inconvenient: they are inhibitory to a great number of enteric bacteria, even *Salmonella*'s growth is observed lightly inhibited due to the use of inhibitors that overcome the growth promoting properties of the nutrients that media contain.

Some of the culture media previously referred, base the *Salmonella* identification on the H_2S production, but this reaction is also characteristic for other Gram-negative organisms, such as *Proteus* and *Citrobacter*.

Other media have been thoroughly marketed for the identification of *E. coli and* coliform bacteria count in several biological samples, such as Endo Agar, Eosin Methylene Blue Agar, MacConkey Agar, Violet Red Bile Agar, among others (Soria Melquizo, F. Difco Handbook. Tenth edition. 1984; MERCK Handbook of Culture Media. 1990; OXOID Handbook of Culture Media. 1995).

These media, in general, contain inhibitors of Gram-positive organisms, which not being in adequate proportion in relation to the nutrients; frequently reduce the growth of target microorganism. On the other hand, in these media is not possible to identify nor *Salmonella* neither the late lactose fermenting organisms.

Diagnostic specificity and/or sensibility of these media generally are not so high, because they base the identification of such organisms on carbohydrate fermenting biochemical

reactions that are common for several target species or genera, as it is the case of lactose degradation by the coliform group (since not all are able to ferment this substrate).

In 1998, Helena Tuompo et al patented a method and a culture medium for the identification of *Salmonella* (Patent No. US 5, 786, 167), based on the ability of this microorganism of metabolize melibiose, mannita and sorbita, as well as on the inclusion of a chromogenic substrate, in order to detect β-galactosidase activity. This medium has the limitation that it does not allow to identify *Proteus* species- an enteric bacteria of sanitary interest, since this organism grows as colorless colonies.

Another inconvenient that presents this medium is that it does not allow the count of enteric bacteria, and *Salmonella* could be confused with mannita fermenting *Shigella* as *S. sonnei* and *S. flexneri.*

That same year Alain Rambach patented a chromogenic-fluorogenic culture medium to detect *E. coli* and the method for its use (Patent No. US 5, 846, 761). The invention consisted in the use of a substrate derived from indolyl-glucuronic acid or it's salts, and a non-chromogenic substrate derived from alkyl, -alkenyl or -aryl of the glucuronic acid or their salts.

This medium is specific for *E.coli* and it does not allow the identification and/or count of *Salmonella* and other microscopic Gram-negative organisms. On the other hand it requires the use of an ultraviolet light lamp, which makes difficult the count.

In the patent No. US 5, 723, 308, Mach et al describe a medium for the rapid count of coliform bacteria. The medium includes a mixture of gelatin peptone and yeast extract, lactose or glucose, sodium chloride, bile salts, arabic gum and pH indicators such as phenol red and sulfonftalein, in quantities from 0,16 to 5 g/ L, in order to identify the bacteria. These high concentrations of indicators are, at the same time, inhibitory and cause the poor or no growth of target microorganisms. The principle of the sugar fermentation, specifically lactose fermentation by coliform organisms, is still being used in this medium with the disadvantage of not allowing the detection of the late fermenting organisms such as, for example *Citrobacter.* Other enteric bacteria are not identified and could be also affected by the high concentration of indicators.

In 1993 Christopher Tate et al patented a very selective medium for *Salmonella* (Patent No. US 5, 208, 150), with the inclusion of Tergitol RTM in a base with xylose-lysine, where sulfapyridine is also included for the inhibition of the *Citrobacter* growth. As for the majority of the media that have been analyzed and that are currently in the market for the *Salmonella* identification, the problem is solved with the inhibition of the flora, which could be confused

with *Salmonella*, mainly *Proteus* and *Citrobacter*. These enteric bacteria, specifically *Citrobacter* is considered a coliform, therefore the count of these organisms seems to be affected in this medium. The use of an antibiotic in the medium, as novobiocin, becomes an inconvenient for the preparation of the medium. Additional cares are needed for its sterilization and conservation.

Alain Rambach patented a medium for the isolation and identification of *Salmonella* (Patent No. US 5, 194, 374), which could be considered the nearest prototype to this invention. It consists in the identification of this organism by means of the splitting reaction of 1, 2-propanodiol in presence of a pH indicator, which is adsorbed in a powdered material, with a particle size of 100 microns. Such material could be silica, silica gel or cellulose. Besides other components, in this medium is included a substrate for β-galactosidase, IPTG and deoxycholates.

The medium previously referred shows the following disadvantages:

- Not all *Salmonellas* "non typhi" give red color, because according to the reports of Monget-Daniel (Patent No. US 5, 434, 056), one strain of each one of the *Salmonella enteritidis, S. gallinarum, S. pullorum and S.paratyphi A* assayed by him, did not developed any color, and one strain of *S. Arizonae* developed a blue color.
- One strain of *Klebsiella pneumoniae* showed a blue color in several tests carried out in the laboratory, when the expected reaction should be violet, taking in account that this organism metabolizes the propane-1,2-diol, it is β-galactosidase positive and the majority of the strains of this organism reveals a blue color after 24 hours.
- In experiments carried out during the development of this invention, the results of Monget were corroborated, finding some strains of *Pseudomonas* with red color, characteristic also for *Salmonella*.
- Unexpected blue color was detected also for strains of *Proteus vulgaris*.
- The medium is not designed for the organisms count, due fundamentally to the composition of nutrients, which do not include the necessary amount of each nutrient in order to overcome the inhibitory power of the deoxycholates included in the formulation. Likewise, the total relationship between nutrients and inhibitors is not enough and does not allow an adequate development of the Gram-negative organisms, and thus finally does not guarantee a sure count of such organisms.
- The previous deficiency shows its negative influence when the Rambach medium is employed for the identification of *Salmonella,* because of the necessity to carry out a

preceding incubation of the sample in a pre-enrichment or enrichment medium, before being inoculated in the Rambach Agar plate. Generally, the period of this enrichment stage extends for 18-24 hours in Selenite Broth, Tetrationate or Rappaport Vassiliadis media.

- In different tests carried out by the applicant of the present invention, strains of *Enterobacter aerogenes* (ATCC 13048) and *Klebsiella pneumoniae* (ATCC 13883), appeared very inhibited in the medium after 18 to 24 hours of incubation at 37 °C. *Proteus vulgaris* (ATCC 13315) and *Proteus mirabilis* (ATCC 7002) did not grow in the same experiment. In another two series of tests, *Enterobacter aerogenes* ATCC 13048 did not grow in the medium, in comparison with experimental media.
- Another disadvantage is the quantity of powdered silica gel that is included in the described medium, it makes difficult its homogeneous distribution and silica easily flocculates, provoking, in many cases, the appearance of a non-adequate consistency of the medium and a troublesome manipulation.
- The high adsorption capacity of the silica does not allow the diffusion of the adsorbed components in the medium.
- The low growth promotion capacity influences even in the growth of *Salmonella*, because in previously carried out experiences, a strain of *Salmonella typhi*, resulted inhibited in the medium.
- The relationship between inhibitors, nutrients and chromogenic substances is not the most appropriate, since it is necessary to include activators of the enzymes (IPTG) in the formula, in order to metabolize the substrates and to develop the reaction in less time.

Disclosure of the Invention

The aim of this invention consists on providing a composition and a method for early detection and differential count of microscopic Gram-negative organisms, allowing, specifically, to identify and carry out a sure and simultaneous detection and/or count of *Salmonella*, *E. coli*, coliforms and other Gram-negative bacteria as: *Pseudomonas*, *Citrobacter* and *Klebsiella*, in early cultivation periods and with a high analytic sensibility.

The novelty of the solution consists on providing mixtures of highly nutritious substances of protein origin, with a high content of total nitrogen- between 10 and 33 %. The content of these mixtures in the formulation is in a relationship from 2:1 to 24:1 to the total content of the inhibitory substances for organisms which have to be inhibited, and more specifically Gram-positives.

On the other hand, in the composition, mixtures of organic and inorganic substances are included, which allows to make a differential count of the target microscopic organisms, being this mixture in an adequate relationship from 0,5: 1 to 2: 1 to the mixture of nutritive substances of protein origin, mentioned before.

The substances of protein origin are selected from several types of compounds, among of them could be mentioned:

- Dehydrated pancreatic or papainic beef heart hydrolysates, with a total nitrogen content from 10 to 15 %, which are in the mixture of substances of protein origin in concentrations from 25 to 75 %.
- Dehydrated enzymatic milk protein hydrolysate with a total nitrogen content from 11 to 20 %, which could be in the mixture in concentrations up to 15 % of said mixture of protein substances.
- Enzymatic microbial autolysates or hydrolysates, with a total nitrogen content from 8 to 15 %, and which are in said mixture in concentrations from 15 to 25 %.
- Mixtures of egg yolk proteins, with total nitrogen content from 15 to 33 %, which could be in the mixture in concentrations up to 45 %.

The composition described in this invention includes small quantities of inhibitors of the growth of Gram-positive microscopic organism, such as cholic and deoxycholic acids as well as bile salts, which are in the composition in a relation between 0.5:1 to 2:1 in respect to the mixture of substances of protein origin, it means in amounts from 0,8 to 4 g/ L.

On the other hand, a novel aspect of the invention consists on the jointly use of compounds previously mentioned, with other compounds, which allow the selective differentiation of target organisms, being the last group of compounds selected from bivalent metal's oxides and siliceous compounds, such as $3MgO \times 4SiO_2 \times H_2O$, $SiO_2\ xH_2O$ and SiO_2. Said substances are in quantities from 2 to 20 g/L (concentrations from 6 to 32% respecting to the total mass of the mixture) together with phenol red or neutral red in quantities from 0,01 to 0,06 g/L (concentrations from 0.03 to 0.18% of the total mass), in presence of alcohols, and said alcohols could be metabolized by the enzymes of some of the target organisms, preferably $C_3H_8O_2$, in quantities from 10 to 14 mL/L and in the presence of chromogenic compounds. Said chromogenic compounds could be metabolized by other microscopic organisms different from those that could metabolize said alcohols, and which, when being metabolized, give to the colonies several colors, different from those colors, characteristic of

the organisms which are able to metabolize said alcohols, preferably X-gal in quantities from 0,05 to 0,1 g/L (concentrations from 0.15 to 0.3% of the total mass of the mixture).

As another novel element in the formulation for this type of medium for the proposed finality, the combination of sodium and magnesium salts, such as magnesium chloride and sodium carbonate, in quantities from 0,01 to 10 g/L was included (concentrations from 0.03% to 32% of the total mass of the mixture). Creatinine and low molecular weight nitrogen compounds could be included in quantities up to 1 g/L (up to 3% of the mixture), sulfured amino acids, such as cystine and cysteine in quantities up to 0,4 g/ L could be also included (concentrations up to 1.25% of the total mass of the mixture).

In the composition, gelling agents are included, preferably agar, with a hardness between 400 and 700 g/ cm^2, in quantities from 13 to 20 g/ L (from 40 to 63% of the total mass of the mixture).

The composition is reconstituted in water in order to be used, in quantities from 30 to 32 g/ L. For this purpose the alcohol is added to the water, shaking until the total distribution. The rest of the ingredients are added to the liquid mixture, shaking and heating to temperature of 100 °C for 1 to 3 minutes and decreasing the temperature to 45-50 °C. The new composition has a pH value from 6,8 to 7,4 (after boiling for 1-15 minutes and cooling until 20-25 °C).

The composition is employed incubating it in a gel form at temperature from 30 to 45 °C, for at least 12 hours.

In this prepared and restored in water composition, was possible to provide a new method for the early detection and differential count of Gram-negative microscopic organisms such as *Salmonella*, *E.coli* and other coliform organisms. *E. coli* and coliforms will show a blue-greenish color of the colony on an orange bottom of the medium. *Salmonella* (non *typhi)* can be detected by a red color on the center of the colonies on a rosy bottom. *Salmonella typhi* and *Proteus vulgaris* could be detected by the transparency of the colonies. *Citrobacter* and *Klebsiella* are differentiated by the violet color of the colony on the rosy or orange bottom of the medium. *Pseudomonas aeruginosa* could be detected by the clear orange color with darker center of the colony, and later becomes greenish after 24 h, and by the production of a yellow-greenish fluorescence under ultraviolet light.

The advantages of the new invention reside in that:

- For first time is provided a composition that allows the early detection and differentiated enumeration of several species and genera of microscopic Gram-negative organisms, among

them *Salmonella typhi*, *Salmonella non-typhi*, *Escherichia coli*, *Proteus*, *Citrobacter* and *Pseudomonas.*

- The balance between the nutritive bases, with adequate total nitrogen content, the relationship between them, and with the inhibitors, facilitates that the target organisms grow without inhibition, especially *Salmonella*, and on the other hand, guarantees that Gram-positive organisms are totally inhibited.

- The diagnostic specificity for the target organisms in the composition is very high, because in all experiences carried out resulted 100 % for all the organisms.

- In an unexpected manner, the isolation and identification of *Salmonella* has been facilitated in only 12 hours, without necessity of the previous pre-enrichment or enrichment of the sample.

- The analytic sensibility is very high, even before 24 hours, for all target organisms.

- With the use of this composition is possible the early identification and enumeration of lactose late fermenting organisms that could be enumerated and identified as coliforms such as *Citrobacter* and *Serratia.*

- It is of great interest that in an unexpected manner *Proteus*, which is an organisms of sanitary interest and which is an enteric bacterium but not a coliform, and *Salmonella typhi*, another Gram negative bacterium, grow in the medium and are identified as colorless colonies, by which, their presence do not interfere in the count of the coliforms, and in this way, they are available for a further identification.

- Species of *Salmonella* have been enumerated in a differentiated form from the mannitol fermenting *Shigella*, such as *Shigella flexneri* and *Shigella sonnei.*

- Due to the balance achieved in the proposed formulation between inhibitors, nutritive bases and other growth promoting substances, and their relationship with the substrates (that allow the differentiated identification from the target organisms) is possible to use low concentrations of the indicators or revealer of the reaction (0,01 to 0,06 g/ L), by which the promoting or nutritive qualities of the product are intensified.

- Low concentrations of indicators are enough for identifying the target organisms, with the saving of these substances, difficult of being obtained by synthesis.

- The substances that conform the composition are thermo-stable and none complicates the preparation of the final medium. They facilitate the preparation of the diagnostic device, without the necessity of sterilizing it.

- The reactions that allow the identification of the target organisms in this composition are clear and common for the strains of each genera or species to identify. None "atypical" responses have been observed, neither for the *Salmonella* nor for the rest of the enteric bacteria, including the coliform group.

- Surprisingly, when using this composition, strains of *Klebsiella* gave a different response to the rest of enteric bacteria, except to *Citrobacter*. This constitutes a new diagnostic possibility because it does allow the presumptive count of these two germs whenever it is required.

- With this invention, a considerable reduction of the total time of the assay is achieved. In the case of *E. coli,* in certain applications, in less than 12 hours could be achieved as result.

- The product does not contain substances difficult to distribute or to dissolve; neither contains incompatible components, by which its preparation is simple.

- The conjunction of several sources of especially selected proteins and proteinaceous substances derived from their hydrolysis, with a nitrogen content from 8 to 33 %, provides enough nutrients for the development of all target genus and species without inhibition in a short period of time. The presence of other components, such as vitamins and microelements favors this balance.

- The invention provides a relationship between the components of organic and inorganic nature and proteinaceuos substances from 0,5:1 to 2:1, which facilitates the differentiation of the target organisms. This relationship allows the growth of the organisms easily inhibited by the group of cholic and deoxycholic acid compounds, and which are difficult to be differentiated by the traditional methods of sugars degradation.

- The addition of bivalent metals oxides and siliceous in quantities from 2 to 20 g/L, besides of activating the microbial metabolism, allows conforming a solid structure, which in conjunction with agar of a selected hardness (400-700 g/cm^2) allow the formation of characteristic colonies. The color of the colonies does not diffuse in the medium, by which finally, facilitates the simultaneous differentiation by colors of a wide range of microscopic organisms.

- The mixture of egg proteins, besides of contributing with nutrients, makes possible, jointly with mentioned in the previous paragraph substances, the appearance of a contrast zone in all the volume of the medium, and this facilitates the detection of several colors and tonalities of the colonies.

- The combination of alcohols degradable by the enzymes of at least one of the organisms to identify, in the established quantities, with the pH indicators and X-gal, facilitates that, at high dilutions of the sample and at few hours from the beginning of the cultivation, (less than 12

hours for *E. coli* and about 12 hours for *Salmonella* and coliforms bacteria), the reactions could be observed and by this mean the right and sure identification is achieved, facilitating the high sensibility and specificity of the diagnostic device.

- The presence of sodium and magnesium salts in predicted quantities, allows to accelerate the enzymatic reactions and, in combination with other compounds such as creatinine and other sulfured amino acids, also offers additional nutrients for target organisms that are susceptible of being inhibited by cholic and deoxycholic acid compounds.

- The quantities in that the composition is restored in water, as well as the preparation method and incubation of the samples, provide an easy, simple and sure procedure for laboratory workers. It offers a superior reliability to the diagnostic procedure.

- The possibility that offers the new composition, for the identification of the microscopic organisms only through their chromatic peculiar characteristics, allows the reading and identification of the results by personnel with a minimum of preparation.

Detailed description of the invention

To prepare the composition described in this invention, the components are prepared according to their physical state: solid or liquid. The preparation of the solid components or powders is described next.

The substances of protein origin selected within the group of pancreatic or papainic beef heart hydrolysate, with a total nitrogen content between 9-14 % and in a quantity from 25 to 77 % concerning the mass of the mixture of said substances of protein origin; enzymatic hydrolysate of milk proteins with a total nitrogen content from 9 to 20 %, and in a quantity from up to 15 % concerning the mass of said mixture, as well as the egg proteins, with a total nitrogen content from 5 to 7 % and in a quantity up to 45 % concerning the mass of the mixture, are sifted or processed in order to achieve the uniformity of the particles size.

These protein origin substances are in a relationship from 2: 1 to 24: 1 in relation to the content of inhibitors, selected within the group of the cholic and deoxycholic acid, bile salts and sodium deoxycholate. They are sifted, being ready to be mixed and homogenized with the rest of organic and inorganic components that are added in a relationship from 0,5: 1 to 2: 1, to the mass of the mixture of protein origin substances.

The pre-mixture is carried out combining bivalent metals oxides and/ or siliceous compounds ($3MgO \times 4SiO_2 \times H_2O$; $SiO_2 \times H_2O$), which have been previously dried and taken in quantities between 6 and 32 % in relation to the mass of the final mixture; pH indicators, preferably phenol red or neutral red in quantities from 0,03 to 0,18 % in relation to the total mixture;

chromogenic compounds that could be metabolized by the action of at least one organism, preferably X-gal in quantities from 0,15 to 0,3 %, concerning the total mass of the mixture. The pre-mixture of these compounds could be grinded and sifted before their homogenization, in a manner that weighing the sum of the quantity of each component in the formulation; an adequate distribution of each one is achieved.

Sodium and magnesium salts (sodium carbonate and magnesium chloride) previously are dried, ground and sifted before adding them to the final mixture in quantities of 0,03 to 32 %.

The nitrogen compound of low molecular weigh, specifically creatinine, (up to 3 % concerning the mass of the final mixture) and the sulfured amino acids preferably cystine and cysteine (up to 1,25% concerning the final mass of the mixture) are sifted before adding them to the total mixture.

The gelling agent, preferably agar, of hardness between 400-700 g/ cm^2, which is used in quantities between 40 - 67 % concerning the final mass of the mixture, is dried off and sifted before adding it to the final mixture.

All components mentioned before, as well as the pre-mixture, are poured together in a homogenizer and mixed until achieving a uniform content with a pH from 6,8 to 7,4. This mixture is poured into flasks and tightly closed, protected from the light, maintaining them at room temperature.

The liquid components of the composition, that could be the polyalcohols, specifically $C_3H_8O_2$, and the egg proteins without dehydration, as well as the distilled or deionized water, are mixed thoroughly until achieving a homogeneous solution.

The powder is added in quantities from 30 to 32 g/L to the liquid mixture, by this way the composition is reconstituted and the suspension of the composition is ready for use.

The composition could be prepared at laboratory scale, weighing the components in separate form within a container and maintaining all the before mentioned proportions. The liquid mixture is added subsequently on the powder mixture. The suspension is agitated and is allowed to swell for 10 minutes before bringing it to boil for a period of 1 to 3 minutes at least. The suspension is cooled down until 45 - 50 °C and distributed in the final assay container. The composition is let to solidify at temperature between 25-30 °C. If humidity in the top of the containers is observed when the content is gelled, it should be dried by any of the well-known methods before the inoculation of the samples. The microscopic organisms, or the samples that contain them, are inoculated by any of the streaking methods, or by any spread

surface methods. The inoculated recipients are incubated to temperature from 30 to 45 °C for at least 12 hours.

The evaluation of the results is carried out observing the color and morphology of the isolated colonies in the surface. The observation of blue-greenish colonies with darker center, on an orange bottom of the medium, regular borders and size of 1 to 5 mm, in dependence of the time of incubation, is characteristic for *E. coli* and coliforms, except *Citrobacter* and *Klebsiella* genera which are observed similar, but with violet color on rosy to orange bottom of the medium.

The *Salmonella* non-*typhi* species are observed with a red color center, on a rosy bottom of the medium, regular borders, and 1 to 6 mm diameter, in dependence of the incubation time.

The colonies of *Salmonella typhi* and *Proteus* are observed with a color characteristic of the medium due to their transparency, regular borders and size of 1 to 3 mm, in dependence of the incubation time.

Strains of *Pseudomonas aeruginosa* are observed with an orange color with dark center and a greenish pigmentation starting from 24 hours of incubation and emitting yellow-greenish fluorescence under low ultraviolet light (365 nm). Other Gram-negatives organisms remain colorless in the medium.

Examples.

Example No. 1.

400 g of the dehydrated composition in powder with the following composition is prepared:

COMPONENT	**G/ 400 G OF MEDIUM**
Pancreatic hydrolysate of beef heart (total nitrogen ≈ 10 %)	63
Enzymatic hydrolysate of milky proteins (total nitrogen ≈ 12 %)	38
Saccharomyces yeast hydrolysate (total nitrogen ≈ 8%)	38

The components related before were previously sifted.

In the composition, the bile salts were included in quality of inhibitors (16,6 g).

Pre-mixture was prepared with 51 g of 3MgO x 4SiO_2 x H_2O, with 1 g of X-gal and 0,4 g of neutral red. All the ingredients were mixed with agar as gelling agent in quantity of 191 g (gel strength of 560 g/ cm^2) and sodium carbonate in quantity of 2 g. When the uniformity was achieved and the pH was adjusted to 7,1, the composition was added into flasks hermetically sealed in 15,7 g quantities.

At the same time the $C_3H_8O_2$ was added in glass flasks for 5 mL.

The content of each flask with the solid composition was poured in an erlenmeyer that contained a mixture of deionized water, and the flask content of $C_3H_8O_2$; the mixture was agitated, leaving it swelling for 10 minutes, then it was heated to boil for 3 minutes, cooled down until the temperature of 45 °C and distributed in Petri dishes. When the composition was gelled, it was carried out the evaluation of its characteristics and functionality. The differentiation of the colonies and the growth promotion in comparison with the Tryptone Soy Agar, was evaluated with certified strains according to the Table No. 1.

Table 1 Differentiation and growth promotion at 24 hours.

Organism	**Strain ATCC**	**Characteristics of isolated colonies**	**Experimental medium CFU/mL**	**Tryptone Soy Agar FCU/mL**	**Signif. Diff. P≤0,05**
Salmonella typhimurium	14028	Red center, regular borders, ≈ 2-3 mm	430 ± 42,4	430 ± 17,6	-
Escherichia coli	25922	Blue greenish color, regular borders, ≈ 2 mm	180 ± 21,2	200 ± 10,6	-
Citrobacter freundii	9080	Violet color, regular borders, ≈ 2 mm	320 ± 98,9	370 ± 3,5	-

Experimental: Composition described in this invention

CFU/ mL: Colony Forming Units for each mL of dilution 10^{-6}

+ Exist significant differences for p ≤0,05

- Do not exist significant differences for p ≤0,05

A dilution 10^{-1} of a strain of *Streptococcus faecalis* ATCC 29212 was inoculated and it was inhibited at 24 hours.

Color diffusion from the colonies to the medium was not observed, which allowed the easy enumeration and differentiation of the colonies. Enumeration and early differentiation of late lactose ferments as *Citrobacter* was achieved. Growth promotion of the assayed species was comparable with a general-purpose medium such as Tryptone Soy Agar, and it was statistically demonstrated. Significant differences between the counts obtained in both media were not achieved. The inhibition of the Gram-positives species was observed still at high concentrations of the inoculum.

Subsequently was proceeded to the evaluation of the new composition in comparison with other media taken as references, these ware:

Brilliant Green Agar (BGA): Prepared at a concentration of 50 g/ L of deionized water, shaking the mixture and boiling until complete dissolution. It was sterilized at 121 °C for 15 minutes, cooled down until 45-50°C and distributed in Petri dishes.

Rambach Agar (RA): The content of a flask (to prepare 1 L) was poured in a flask with 1 L of the mixture of the liquid supplement with deionized water. It was shaken and heated with agitation until boil, cooled down until 45-50 °C and distributed in Petri dishes.

Violet Red Bile Agar (VRBA): Prepared at a concentration of 38,5 g/ L in deionized water, it was shaken and heated with agitation until boiling, cooled down until 45-50 °C and distributed in Petri dishes.

According to Table No. 2, besides the growth of the microorganisms in the medium, was compared the growth promotion at a dilution 10^{-6} of each inoculum. .

Table 2 Evaluation of the growth different microorganisms (incubation at 37 °C for 24 h).

Organism	Medium	Characteristic and color of the isolated colonies	Growth promotion	
			CFU/mL	Signif. Diff. p≤0,05
Salmonella enteritidis ATCC 13076	VRBA	Pink, regular borders, ≤ 1 mm	15 ± 7,6	-
	RA	Violet red, regular borders, ≈ 1-2 mm	100 ± 40,0	
	Experimental	Red center, regular borders ≈ 1-2 mm	130 ± 74,0	
Salmonella typhimurium ATCC 14028	VRBA	Pink, regular borders, ≤ 1 mm	50 ± 36,5	-
	RA	Violet red, regular borders, ≈ 1-2 mm	260 ± 201,5	
	Experimental	Red center, regular borders ≈ 1-2 mm	220 ± 138,0	
Salmonella typhi ATCC 19430	VRBA	No growth	No growth	+
	RA	No growth	No growth	
	Experimental	Colorless, regular borders, ≈ 2 mm	130 ± 28,7	
Proteus vulgaris ATCC 13315	VRBA	No growth	No growth	+
	RA	No growth	No growth	
	Experimental	Colorless to pink regular borders ≈ 2 mm	225 ± 28,0	

Experimental: Composition described in this invention

CFU/ mL: Colony Forming Units for each mL of 10^{-6} dilution

\+ Exist significant differences for p ≤0,05

\- Don't exist significant differences for p ≤0,05

The growth of *Proteus vulgaris* in the medium is observed even at high dilutions, the differentiated growth of *Salmonella* non *typhi* species is demonstrated with intense pink to red color in the center of the colonies, well differentiated from the strain of *Salmonella typhi.* The last one grew without inhibition in the composition, even at high dilutions.

Three strains of the coliform group organisms were also inoculated in the composition, using reference traditional media: Violet Red Bile Agar, and Rambach Agar. The growth and characteristic of the colonies are shown in the Table No. 3.

Table No. 3 Evaluation of the growth of several microorganisms of the coliform group in the composition.

Organism	**Medium**	**Characteristic and color of the isolated colonies**	**Growth promotion**	
			CFU/mL	**Signif.diff. p≤0,05**
Enterobacter aerogenes ATCC 13048	VRBA	Pink, regular borders ≈ 2 mm	350 ± 130,5	-
	RA	No growth	No growth	
	Experimental	Blue-greenish, regular borders, ≈ 2-3 mm	60 ± 19	
Escherichia coli ATCC 25922	VRBA	Red with bile precipitate, regular borders, ≈ 3 mm	395 ± 116	-
	RA	Dark blue, with blue halo in the medium, regular borders, ≈ 2 mm	135 ± 115	
	Experimental	Blue-greenish, regular borders, ≈ 2-3 mm	270 ± 43	
Citrobacter freundii ATCC 9080	VRBA	Gray, regular borders, ≈ 2 mm	765 ± 328	-
	RA	Red maroon, regular borders, ≈ 1-2 mm	305 ± 20	
	Experimental	Pink-violet intense, regular borders, ≈ 2 mm	460 ± 7,5	

Experimental: Composition described in the present invention

CFU/ mL: Colony Forming Units for each mL of 10^{-6} dilution

\+ Exist significant differences for p ≤0,05

\- Do not exist significant differences for p ≤0,05

The growth promotion of the assayed coliform microorganisms was statistically similar to that obtained in traditional medium (Violet Red Bile Agar) and higher than the obtained in the Rambach Agar for *Enterobacter aerogenes.* A differentiation of this coliform group was achieved in the experimental composition by their characteristic blue-greenish color, differing

from the *Citrobacter* species by their violet color. The differentiation of this late fermenter organism is not achieved in traditional media based on the lactose fermentation.

The composition was evaluated with certified strains, and the incubation time was set for 18 hours, according to the data showed in the Table No. 4.

Table No. 4. Characteristic of the growth and the colonies at 18 hours

Organism	Strain	Characteristic of the colonies
Shigella flexneri	ATCC 12022	Transparent, regular borders, less than 1 mm
Salmonella typhi	ATCC 19430	Transparent, regular borders, brilliants
Salmonella typhimurium	ATCC 14028	Red centers, regular borders
Proteus vulgaris	ATCC 13315	Transparent, regular borders
Staphylococcus aureus	ATCC 25923	Inhibited
Escherichia coli	ATCC 25922	Blue greenish, regular borders, brilliants
Pseudomonas aeruginosa	ATCC 27853	Clear oranges, darker center, regular borders, greasy aspect
Citrobacter diversus	sp.	Violets, regular and transparent borders, darker center
Klebsiella pneumoniae	ATCC 13883	Violets, regular borders
Enterobacter aerogenes	ATCC 13048	Blue-greenish, regular borders, different from *E. coli* (more clears)

A good differentiation without enrichment at 18 hours was achieved. The strain of *Pseudomonas aeruginosa* was correctly identified, as well as the strains of *Klebsiella* and *Citrobacter*. On the other hand, *Staphylococcus aureus* was totally inhibited in the medium.

The composition was also evaluated in chicken samples contaminated with *Salmonella*. The sample was cut in pieces (360 g) and putted in a polyethylene bag and buffered peptone water was added. The content was agitated, and the inoculum was taken. Serial dilutions were prepared from 10^{-1} until 10^{-7}. Two dishes were inoculated, the first one with the more concentrated sample and the second with the minor concentration. Both dishes were incubated for 24 hours at 37 °C. The results are shown in Table No. 5.

Table No. 5. Characteristic of the colonies growth at 24 hours of incubation.

Charact ristic of the obs rv d colonies	**Organisms id ntifi d by the n w composition**
Red colonies, regular borders, darker center, ≈ 2 mm	*Salmonella* sp.
Red colonies, irregular borders, wide and clear halo	*Salmonella sp.*

At 24 hours, there is a good identification of *Salmonella* without the pre-enrichment or enrichment stages.

An evaluation in 20 samples of *Salmonella* contaminated foods was carried out. A comparison of several combinations of enrichment media and traditional solid media for the identification and isolation of *Salmonella* was carried out, with direct inoculation of the sample in the composition described in this invention and in a parallel experiment, with this proper composition in combination with several enrichment broths. The results are described in Table No. 6.

The media used in the experiment were:

Tetrationate Broth: Prepared by dissolving 46 g of the powder in 1L of deionized water, shaking the mixture until the complete homogenization, and adding a solution of iodine, prepared especially for this medium. It was heated to boiling during 1 minute and distributed into tubes in quantities of 10 mL.

RAMBACH Medium: The content of a flask (to prepare 1 L), was poured in 1 L of a mixture of liquid supplement with deionized water. It was agitated and heated with agitation until boiling, cooled down until 45-50 °C and was distributed in Petri dishes.

Hektoen Enteric Agar: Prepared at a concentration of 75,7 g/L of deionized water. It was shaken and heated with agitation until boiling (1-3 minutes), cooled down until 45-50 °C and distributed in Petri dishes.

Rappaport Vassiliadis Broth: Prepared at a concentration of 30 g/L of deionized water. It was agitated and heated with agitation until boiling (1-3 minutes), was sterilized by autoclaving at 121 °C for 15 minutes, cooled down until 45-50 °C and distributed into tubes.

Table No. 6. Comparison between the experimental and the traditional media and methods for isolation and identification of *Salmonella*

Methodology	False positive results
Traditional (TT/HE)	11
Traditional (TT/RAM)	9
TT/ Experimental	7
Traditional (RV/HE)	4
Traditional (RV/RAM)	4
RV/ Experimental	2
Experimental (without enrichment)	0

Experimental: Composition described in the present invention

HE: Hektoen Enteric Agar

RV: Rappaport Vassiliadis Broth

RAM: Rambach Medium

TT: Tetrationate Broth

The number of false positive results in all cases was significantly less in the composition and method described in this invention than in the case of traditional media and methods. The best results were obtained in the case of the direct inoculation of the samples on the composition. With this composition, no false positives where obtained.

Example No. 2.

The composition was prepared weighting the ingredients separately directly to an erlenmeyer according to the example 1. The sulfured amino acid L-cystine also was added to the composition in quantity of 0,2 g/ L.

A mixture of solid ingredients of the composition was mixed with a mixture of deionized water and $C_3H_8O_2$. The further preparation, including the solidification (gelling) was executed as described in the example 1.

Certified strains of *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Citrobacter freundii* (ATCC 9080) and *Streptococcus faecalis* (ATCC 29212) were inoculated in the composition by streaking in the surface of the gel, until achieve isolated colonies.

A red intense color in the colonies of *Salmonella* at 24 hours was observed and thus made easy the differentiation of this organism even at 21 hours of incubation. The strain of *E. coli* showed blue-green color, different from the other inoculated coliform (*Citrobacter freundii),* which was observed with characteristic violet color colonies. It was evident the inhibition of

the strain of *Streptococcus faecalis,* who didn't show growth in any of the tests, even in the case of streaking with concentrated inoculum in 10^{-1} dilution.

Example No. 3.

The composition was prepared with the ingredients described in the example 1, with the difference that the heart hydrolysate used was obtained by a papainic hydrolysis (total nitrogen ≈ 12 %). The concentration of this ingredient was similar to the example 1. Another difference consist on the use sodium deoxycholate as inhibitor at a concentration of 1 g/L, and the chromogenic substrate used was x-gal, added in 33 % less concentration than that described in the example 1. These ingredients were weighed in an erlenmeyer flask.

A mixture of deionized water and $C_3H_8O_2$ was added to the mixture of the previously described solid ingredients and further the composition was prepared according to the method showed in the example 1.

Certified strains of *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Proteus vulgaris* (ATCC 13315), *Salmonella enteritidis* (ATCC 13076), *Enterobacter aerogenes* (ATCC 13048), *Salmonella typhi* (ATCC 19430) and *Proteus mirabilis* (ATCC 7002) were inoculated by streaking in the surface to obtain isolated colonies. The results showed in the Table No. 7 evidence the correctly differentiation of the inoculated species at 18 hours of incubation.

Table No. 7. Characteristics of the medium and the colonies at 18 hours of incubation.

Organism	Color of the medium	Color and charact ristic of the isolated colonies
Escherichia coli	Orange	Blue, regular borders, convex, size ≈ 2 mm
Salmonella typhimurium	Orange-reddish	Red center, regular borders, convex, size ≈ 1,5 - 2 mm
Proteus vulgaris	Orange	Colorless, regular borders, size ≈ 1,5 mm
Salmonella enteritidis	Orange-reddish	Red center, regular borders, convex, size ≈ 1,3 mm
Enterobacter aerogenes	Orange	Clear blue, regular borders, convex, size ≈ 2 mm
Salmonella typhi	Orange	Colorless, regular borders, convex, size ≈ 1,5 mm
Proteus mirabilis	Orange	Colorless, regular borders, size ≈ 1,5 mm

An early and differentiated growth of the species of *Salmonella* non-*typhi* was observed due to the rosy intense color. The strain of *Salmonella typhi* was colorless in the medium and it was smaller in size than the rest of *Salmonella*. The growth of the *Proteus* species was observed, evidencing that this organism is not inhibited, which is a characteristic that differentiates this composition from the rest of the majority of the media distributed already in the market for the differentiation of *Salmonella*.

Example No. 4.

The composition was prepared according to the example 3, but with the addition of dehydrated egg yolk in quantities of 4 g/ L (V1) and 8 g/ L (V2). The method of preparation was similar to that described in the example 3.

Certified strains of *E. coli* (ATCC 25922), *Salmonella typhi* (ATCC 19430), *Proteus vulgaris* (ATCC 13315) and *Streptococcus faecalis* (ATCC 29212) were inoculated by streaking in the gel surface. The observation of the growth was carried out after 24 hours of incubation, and the results at 24 hours are described in the Table No. 8.

Table No. 8. Functionality of the composition with egg yolk

Organism	Variant	Color of the medium	C l r and morphol gy of th isolated colonies
Escherichia coli	V1	Orange, opaque	Blue greenish, regular borders, convex, size ≈ 2 mm
	V2	Orange, opaque	Blue greenish, regular borders, convex, size ≈ 2 mm
Salmonella typhimurium	V1	Orange-yellowish, opaque	Red center, regular borders, convex, size ≈ 1-2 mm
	V2	Orange-yellowish, opaque	Red center, regular borders, convex, size ≈ 1-2 mm
Klebsiella pneumoniae	V1	Rose-orange, opaque	Violet, regular borders, convex, size ≈1-2 mm
	V2	Rose-orange, opaque	Violet, regular borders, convex, size ≈1-2 mm
Salmonella typhi	V1	Orange, opaque	Colorless to pinks, regular borders, convex, size ≈ 1 mm
	V2	Orange, opaque	Colorless to pinks, regular borders, convex, size ≈ 1 mm
Proteus vulgaris	V1	Orange, opaque	Colorless, regular borders, convex, size ≈ 1 mm
	V2	Orange, opaque	Colorless, regular borders, convex, size ≈ 1 mm
Streptococcus faecalis	V1	Orange, opaque	Small colonies, very inhibited, size ≈ 1 mm
	V2	Orange, opaque	Small colonies, very inhibited, size ≈ 1 mm

The composition according to this invention showed a great opacity in the two variants, and thus facilitated a good contrast for the observation of the microorganism's growth in the gel surface. A differentiation of *Klebsiella pneumoniae* from other coliforms was also observed due to the violet color of the first, thanks to the degradation of the chromogenic substrate and the fermentation of $C_3H_8O_2$, in presence of a pH indicator. A growth of the *Proteus* species was also observed.

The *Salmonella* species were differentiated also at 24 hours of incubation in the experimental composition, while the growth of *Streptococcus faecalis* was imperceptible at 24 hours.

Example No. 5.

The composition was prepared according to that described in the example 3, with the further addition of magnesium chloride in quantity of 5 g/L. The method of preparation was similar to the example 3.

Certified strains of *E. coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Salmonella typhi* (ATCC 19430), *Salmonella enteritidis* (ATCC 13076), *Streptococcus faecalis* (ATCC 19433), were inoculated by streaking in the gel surface. The observation of the results was executed at 24 hours of incubation and is described in the Table No. 9.

Table No. 9. Characteristics of the medium and the colonies.

Organism	Color of the medium	Color and characteristic of the isolated colonies
Escherichia coli	Orange	Blue greenish, regular borders, convexes, size ≈ 2 mm
Salmonella typhimurium	Orange-reddish	Red center, regular borders, convex, size ≈ 2 mm
Salmonella enteritidis	Orange-reddish	Red center, regular borders, convex, size ≈ 2 mm
Salmonella typhi	Orange	Colorless, regular borders, convexes, size ≈ 1,5 mm
Streptococcus faecalis	No growth	No growth

A good growth and an early differentiation of *Salmonella* non-*typhi* species were observed due to their red center. The strain of *E. coli* resulted, as expected, showing blue color of the isolated colonies. The strain of *Streptococcus faecalis* was inhibited in the composition, evidencing their inhibitory power for the Gram-positives organisms.

Example No. 6.

The composition was prepared with the addition of the nitrogen compound creatinine, in quantity of 0,5 g/ L. The ingredients were weighed in an erlenmeyer.

A mixture of deionized water with $C_3H_8O_2$ was added to the mixture of solid ingredients and prepared according to the example 1.

A certified strain of *Salmonella typhimurium* (ATCC 14028) was evaluated. It was inoculated by streaking on the gel surface, until obtaining isolated colonies.

An abundant growth of organisms with red color center was achieved at 24 hours of incubation, and at 36 hours changed to dark red, due to the addition of creatinine in the composition.

Example No. 7.

The evaluation of the composition was carried out with the formulation described in the example 3, evaluating samples of contaminated foods. The detection of *Salmonella* in contaminated meat was carried out in parallel by the traditional method and using the new composition, without pre-enrichment.

Dilutions of the samples were inoculated directly in the surface of the composition by streaking, to obtain isolated colonies. The presence of *Salmonella*, coliforms and other enteric bacteria was detected in 24 hours. For the traditional method, the sample was incubated in peptone buffered solution (24 hours to 37 °C), it was enriched in Tetrationate Broth (24 hours at 43°C) and subsequently it was inoculated in Brilliant Green Agar (prepared according to example 1). The presumptive result for the detection of the presence of *Salmonella* by the traditional method was observed only after three to four days, due to this set of steps.

The suspicious colonies obtained by both methods were picked up and were submitted to the corresponding biochemical tests. The colonies were inoculated in 3 tubes in the media for biochemical analysis (Kligler Iron Agar, Urea Agar, Tryptone Broth and for Indol test in Lysine Iron Agar). The presence of *Salmonella* by both methods was confirmed, achieving a reduction of the total time of the analysis with the new composition of, at least, three days.